Indian Journal of Experimental Biology
Vol. 57, November 2019, pp. 796-805

Induction of systemic acquired resistance in *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. by an endophytic bacterium antagonistic to *Phytophthora meadii* McRae

Amith Abraham[1,2#], Shaji Philip[1], Sunilkumar P Narayanan[3], Kuruvilla C Jacob[1], Raveendran Sindhu[4], Ashok Pandey[5], Byoung-In Sang[2] & Kochupurackal Jayachandran[6]*

[1]Rubber Research Institute of India, Kottayam, Kerala, India

[2]Department of Chemical Engineering, Hanyang University, 222 Wangshimni-ro, Seongdong-gu, Seoul 04763, Republic of Korea

[3]Institute of Biophysics and Physical Biochemistry, Centre of Magnetic Resonance in Chemistry and Biomedicine (CMRCB) University of Regensburg, D-93040 Regensburg, Germany

[4]Microbial Processes and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram-695 019, Kerala, India.

[5]Centre for Innovation and Translational Research, CSIR-Indian Institute of Toxicology Research (IITR), 31 MG Marg, Lucknow-226 001, Uttar Pradesh, India

[6]School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India

Received 02 January 2019; *revised 19 March 2019*

Abnormal leaf fall (ALF) disease caused by *Phytophthora meadii* McRae is one of the limiting factors of growth and latex yield in *Hevea brasiliensis* (Willd. ex A.Juss.) Müll.Arg. Endophytic bacteria residing within the host plant offer very high application potential as biocontrol agents for ALF disease management. Screening of bacterial endophytes from leaf, petiole and root tissues of *Hevea brasiliensis* was done for the selection of a potent antagonistic isolate against *P. meadii*. The most efficient antagonistic isolate was identified as *Alcaligenes* sp. The biochemical estimation of antagonist treated plants showed induction of peroxidase activity in leaves. Quantitative PCR analysis confirmed higher activity of lignin biosynthesis promoting class III peroxidase transcripts in antagonist treated plants of *H. brasiliensis*. Homology model of the peroxidase class III protein was built from the deduced amino acid sequence and was found to carry 36% helical and 5% beta sheets. Computer aided docking studies of the substrates (p-coumaryl, coniferyl and sinapyl alcohol) with the target proteins showed that the protein-substrate complexes were stabilized by various interactions and proposed that precursors of lignin biosynthesis were preferred by induced peroxidase in *Hevea brasiliensis* for lignifications. The present study revealed the potential of an antagonistic endophyte *Alcaligenes* sp. EIL-2 for inducing class III peroxidase involved in defence mechanism in *Hevea brasiliensis.*

Keywords: Abnormal leaf fall (ALF), Bacterial endophytes, Biocontrol, Coniferyl, Coumaryl, Latex, Lignifications, Rubber leaf drop, Rubber tree, Sinapyl

Biological control of plant pathogens, particularly use of microbial agents, is an emerging strategy to fight plant diseases. They induce defence system to produce antibiotic to antagonise pathogens[1]. Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant's innate defences are activated and effective against a broad range of pathogens[2]. The major model in research on evolution of induced resistance in crop plants is gene-for-gene co-evolution[3]. SAR (Systemic Acquired Resistance) is one form of induced resistance that can be triggered by exposing the plant to virulent, avirulent, and non-pathogenic microbes or artificially with chemicals, such as salicylic acid, 2,6-dichloro-isonicotinic acid (INA) or benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH)[4]. SAR is characterized by an early increase in endogenous salicylic acid (SA) and accumulation of cell wall lytic enzymes[5]; the synthesis of phytoalexins[6]; the reinforcement of cell walls by increase in peroxidase activities and the deposition of lignin and callose[7].

Production of siderophores or salicylic acid by biocontrol agents can induce systemic resistance in plants similar to SAR[8,9]. Studies reported that the biological control agent, *Bacillus mycoides* isolate

*Correspondence:
Phone: +91 9446356612 (Mob.)
E-mail: jayansbs@gmail.com

Bac J (BmJ), is capable of inducing systemic resistance in sugar beet[10]. Saravanakumara *et al*.[11] reported that protection of tea against *Exobasidium vexans* induced by a bacterial suspension of *Pseudomonas fluorescens* Pf1 was followed by the induction of chitinase, β-1,3-glucanase, polyphenol oxidae and peroxidase. Park & Kloepper[12], reported that *Bacillus* sp. induce *PR-1a* gene in tobacco. A local host-defence reaction was induced by an endophytic *Burkholderia* strain in *Vitis vinifera* plants[13].

The inducible defence responses were mainly mediated through various Pathogenesis Related (PR) proteins. The involvement of PR proteins in enhancing plant resistance to pathogens has been reported by Metraux & Boller[14], and over-expression of a variety of PR genes in transgenic plants enhances their resistance to various fungal pathogens[15]. To date, there is no reliable, systematic approach for identifying novel SAR-eliciting biological control agents. Therefore, the numbers of organisms with this mode of action are belittled in the pool of available biological control agents. However, SAR is associated with pathogenesis-related (PR) proteins, known molecular markers that could be exploited for identification of inducers[16].

Here, we screened and isolated an antagonistic bacterial endophyte against *Phytophthora meadii* McRae and tried to understand systemic acquired resistance in *Hevea brasiliensis* by induction of pathogenesis related (PR) proteins prior to disease challenge assays.

Materials and Methods

Isolation and screening of antagonistic bacterial endophytes against *Phytophthora meadii*

Freshly collected root, petiole and leaf samples of *H. brasiliensis* were cut into small pieces (1.0 g of wet wt.) and were surface sterilized by 2% (v/v) sodium hypochlorite (Merck, India) for 3 min followed by five rinses in sterilized distilled water. All samples were homogenised with mortar and pestle, 1.0 mL homogenate was serially diluted with 9.0 mL sterile 0.85% NaCl up to10^{-6} dilution, plated onto Tryptic Soy Agar (Hi Media Laboratory Pvt. Ltd., India) and incubated for 48 h at 28±2°C. The individual colonies of differing morphologies were picked and re-streaked on fresh plates to obtain pure cultures. The endophytic bacterial isolates were screened for their ability to inhibit the growth of the pathogen, *P. meadii*. Isolates were assessed by dual culture technique using Potato Dextrose Agar (PDA) plates. The PDA plates inoculated with *P. meadii* were served as the control. After seven days of incubation at 28±2°C, colony diameters and inhibition zones were measured. The percent growth inhibition was calculated using the formula $n=(a-b)/a\times100$, where n is the percent growth inhibition, a is the colony area of uninhibited *P. meadii* and b is the colony area of treated *P. meadii*.

Identification of antagonistic endophyte

The endophytic bacterial isolate showing maximum % growth inhibition against *P. meadii* was identified on the basis of sequence analysis of 16S rRNA gene. The conserved eubacterial primers used for amplification of 16S ribosomal DNA were (1) pA- 5'-AGAGTTTGATCCTGGCTCAG-3'; (2) pH- 5'-AAGGAGGTGATCCAGCCGCA-3' and the final concentration of the reagents were 1.0 mM $MgCl_2$, 200 μM dNTP, 100 pmol primers and 50 ng DNA. The PCR reaction was carried out in eppendorf AG22331 Thermal Cycler with the following PCR cycle: one cycle at 94°C for 2 min, followed by 35 cycles at 94 and 55°C for one min each, and 72°C for 2 min, followed by final 2 min incubation at 72°C. The PCR products were size fractionated on 1% agarose gel and the bands were excised from the gel and purified using GenElute™ Gel Extraction Kit (Sigma Aldrich, Germany). Purified 16S rDNA sequences were cloned in pGEMT Easy vector (Promega, USA), transformed in JM 109 cells (Promega, USA) and sequenced at Macrogen, Korea. The sequence similarity was analysed by sequences available in the National Centre for Biotechnology Information (NCBI) database using BLAST analysis and isolates were identified on the basis of the best match in the database. Sequences of antagonistic bacterial endophyte and reference sequences from NCBI GenBank were aligned using the multiple sequence alignment program ClustalW2. Using the alignment file generated by ClustalW2, phylogenetic analysis was performed in MEGA4[17]. UPGMA[18] was used to infer the phylogeny across the data. Bootstrap analysis (1000 replicates) was also performed to check the reliability of the phylogram[19].

Biochemical estimation of PR Proteins (β-1,3-Glucanase, Peroxidase and Phenylalanine ammonia lyase) in antagonist inoculated and control plants of *H. brasiliensis*

The biochemical changes during interaction of antagonist with *H. brasiliensis* (clones RRII 105) were

studied in one year old polybag plants grown in greenhouse. The selected antagonist was inoculated in Tryptic Soya Broth (TSB) and incubated at 28±2°C for three days with constant shaking at 180 rpm, yielding 10^8 CFU/mL. Bacterial broth was diluted with water (1:5) and plants were inoculated by foliar spraying (50 mL/plant) and soil application (50 mL/plant). The treatments included: (i) untreated control; (ii) application of media alone; and (iii) application of antagonist broth. Each treatment was applied to six plants arranged in completely randomized design (CRD). Mature leaves of antagonist treated and control plants were collected at 0, 48, 96 and 144 h and tissues were stored in deep freezer (−80°C) until used for biochemical analysis. The activity of β-1,3-glucanase[20], peroxidase[21] and phenylalanine ammonia lyase[22] were estimated among different treatments. β-1,3-glucanase activity was expressed as μg glucose released min^{-1} g^{-1} tissue. Peroxidase and phenylalanine ammonia lyase activity was expressed as U/L and nmol *trans*-cinnamic acid min^{-1} g^{-1} tissue, respectively.

Expression analysis of peroxidase gene from antagonist inoculated and control plants of *H. brasiliensis*

RNA isolation and cDNA synthesis

Total RNA was isolated from the induced leaves of the clone RRII 105 at 48 h using Spectrum Plant Total RNA Kit (Sigma). The RNA was checked in agarose gel electrophoresis and the purity was estimated in NanoDrop ND-1000 spectrophotometer (USA). Total RNA from the leaves were used as template for synthesis of cDNA using RT-PCR kit (Improm-II Reverse Transcription System, Promega, USA). About 1.0 μg of total RNA was combined with 0.50 μg oligo (dT) primers and the tube was incubated for 5 min at 70°C and immediately chilled in ice for 5 min. The 15 μL reverse transcription reaction mixture was prepared using 4 μL buffer supplied by the manufacturer along with 1.5 μL $MgCl_2$ (1.8 mM), 1 μL of deoxyribo-nucleotide triphosphate (dNTP) mix (0.05 mM of each dNTP) and 1 μL reverse transcriptase. The RNA-Primer mix was added to the reaction mix and incubated at 25°C for 5 min for proper annealing. cDNA synthesis was allowed to occur at 42°C for 60 min. After one hour, the reaction was inactivated by heating at 70°C for 5 min and the mix (20 μL) was subsequently diluted to 200 μL with sterile deionized water and stored at −20°C.

qPCR analysis

To quantify peroxidase gene expression, qPCR was performed in Roche Real Time Thermal Cycler (Light Cycler 480 II). Primers were designed (amplicon size of 100-200 bp) from the *H. brasiliensis* class III peroxidase gene deposited in the NCBI GenBank accession (GenBank ID.DQ 650301) using the Primer Express software (Version 3.0). The house keeping gene, β-actin, was used as the internal control for normalization. The peroxidase and β-actin primer sequences were as follows β-actin Forward 5'-CTCTTCCACATGCCATCCT CC-3'; β-actin Reverse 5'-GTTTCCAGT TCCTGCTCATGA-3'; Peroxidase Forward 5'-AATTGGCACGAATTTCCC-3'; Peroxidase Reverse 5'-CACGGATAAGAGAACA AGG-3'. Real time PCR was conducted using SYBR Green PCR Master mix in a 20 μL master mix reaction containing 1 μL of 1:10 diluted cDNA and 50 nM of each primer. The PCR conditions were as follows: initial step of 95°C for 7 min, followed by 40 cycles of 95°C for 15 s, 55°C for 1 min and 72°C for 1 min. This step was followed by a melt curve analysis (95°C for 15 s, 60°C for 1 min and 95°C for 30 s). Reaction efficiency of both target genes and the endogenous control was calculated based on the formula, $E= 10^{(-1/slope)} -1$. Each treatment consisted of three biological replicates and each biological replicate had three technical replicates. Negative controls without the cDNA template were run to assay for false positive signals. Data analysis was performed using the Smart Cycler software (version 2.0d). Real time PCR results were expressed as Ct (cycle threshold) values. The Relative Quantification (RQ) values were used to study the fold change in the expression level between the treatments. RQ values were calculated by comparative Ct method ($RQ = 2^{-\Delta\Delta Ct}$, ΔCt = Ct of target gene – Ct of endogenous control, ΔΔCt = ΔCt of treatment – ΔCt of control). Melt curves which were unique for each gene of interest were used to identify the specific PCR products.

***In silico* studies of class III peroxidase protein and its possible interaction with substrates (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) in lignin biosynthesis pathway**

Amino acid sequence and physicochemical properties

The amino acid sequence of peroxidase protein was obtained from nucleotide sequence of peroxidase gene (NCBI GenBank ID: DQ650301) by translation using the EXPASY tool (http://web.expasy.org/translate/). Physicochemical properties like molecular weight, isoelectric point (pI), half-life, aliphatic index, amino acid property, instability index and Grand Average Hydropathy (GRAVY) were obtained by the EXPASY tool ProtParam (http://web.expasy.org/protparam/).

Secondary structure elements of peroxidase protein were predicted using the PredictProtein Server (http://web.expasy.org/predict protein/). Amino acid sequences of peroxidase from related plant species were aligned with *H. brasiliensis* sequence using the multiple sequence alignment program ClustalW2. Using the alignment file generated by ClustalW2, phylogenetic analysis was performed in MEGA4[17]. UPGMA[18] was used to infer the phylogeny across the data. Bootstrap analysis (1000 replicates) was performed to check the reliability of the phylogram[19]. Extracellular nature of the protein was predicted by Signal-P programme[23].

Homology modeling

The three-dimensional structure of peroxidase was homology modelled using the SWISS-MODEL workspace, which is an integrated web-based modelling tool[24]. The template used for modelling was the peroxidase (PDB ID: 1pa2A) from *Arabidopsis thaliana*. Energy minimization of the built model was done using the GROMOS96 implemented in the Swiss PDB viewer program[25]. Quality of the predicted model was analyzed by PROCHECK[26]. Visualization and analysis of the model were done using the Swiss PDB viewer and PyMOL programs[27]. The active site was identified using the online Q-site finder tool to predict possible binding sites[28].

Docking studies of modelled peroxidase with substrates of lignin biosynthesis pathway

Computer aided molecular docking of the substrates (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) to the active site of the modelled structure of peroxidase was done using Autodock4.2 program[29]. The program AutoDock allows consistent computational docking of flexible ligands and receptor with a maximum 32 torsional degrees of freedom. For the best placement of the ligand, Lamarckian genetic algorithm was used in the program. Initial co-ordinates of the substrates were obtained from the PDB database. The modelled structure of the peroxidase was taken as receptor protein. Prior to docking, the bound ligand and water molecules were removed from the template crystal structure. The missing hydrogens were added to the ligand and receptors. Docking parameters modified from the defaults were: number of individuals in the population (set to 300), maximum number of energy evaluations (set to 2500000), maximum number of generations (set to 27000) and number of hybrid GA-LS runs (set to 100). The ligand was initially placed next to the active site of receptor. Flexible docking strategy was applied in which all the binding site residues were set flexible. All rotatable bonds in the ligands were allowed to rotate during the docking trials. For each of the substrate-receptor complexes, 100 independent docking runs were calculated and the lowest energy pose with acceptable geometry was selected, for further analysis.

Statistical analysis

The data on biochemical estimation of PR Proteins and peroxidase gene expression analysis were analysed by analysis of variance (ANOVA) and treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was SPSS version 10.0.

Results

Isolation and screening of antagonistic bacterial endophytes against *P. meadii*

A total of 252 morphologically different bacterial endophytes were isolated as representative of the different populations. Of the 252 bacterial isolates evaluated for the *in vitro* inhibition, 42 isolates resulted in suppression of *P. meadii* with mycelial inhibition ranging from 15 to 62.5%. The isolate EIL-2 strongly inhibited the growth of *P. meadii* and showed highest percent of mycelia inhibition (62.5%). The isolate EIL-2 was used for further studies.

Identification of antagonistic endophyte

Data from molecular and phylogenetic analyses were used to characterize taxonomically the selected antagonistic bacterial endophyte from *Hevea brasiliensis*. PCR amplification of the 16S rDNA generated a fragment of approximately 1.6 kb and sequence of the isolate was compared to the sequences of organisms represented in the database GenBank. The isolate showed 99% identity to *Alcaligenes* sp. The highest score sequences were recovered from the database as reference sequences and aligned with the 16S rDNA sequence of the endophytic isolate from *H. brasiliensis*. Phylogenetic analysis was conducted in MEGA4 based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. Evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site (Fig. 1).

Biochemical estimation of PR proteins- in antagonist inoculated and control plants of *H. brasiliensis*

Activities of PR proteins, such as β-1, 3-glucanase, peroxidase and phenylalanine ammonia lyase (PAL), were estimated among different treatments and there was no statistically significant variation of β-1,3-glucanase and PAL activity among antagonist inoculated and control plants of *H. brasiliensis*. The β-1,3-glucanase activity was 30-37 μg glucose released min^{-1} g^{-1} tissue in antagonist inoculated and control plants. Phenylalanine ammonia lyase activity in antagonist treated and control plants were 40 nmol *trans*-cinnamic acid min^{-1} g^{-1} tissue. Foliar and soil application of antagonistic bacteria induced the plants to synthesize more peroxidase enzyme in leaf tissues of *H. brasiliensis*. Maximum enzyme activity was 134 U/l in antagonist inoculated leaf tissues of *H. brasiliensis* at 96 h of application (Table 1).

Expression analysis of peroxidase gene from antagonist inoculated and control plants of *H. brasiliensis*

The good quality total RNA was isolated from the leaf tissues and expression level of peroxidase gene in clone RRII 105 was estimated by quantitative PCR analysis. Amplification curves with Ct values and melting peaks showed the quality of the amplification process in qPCR. Relative quantification values were analyzed and antagonist inoculated plants showed 2-fold increase in the expression of class III peroxidase gene compared to controls. Gel photographs from the qPCR amplified product also confirmed the increased expression of peroxidase gene in antagonist inoculated plants (Fig. 2).

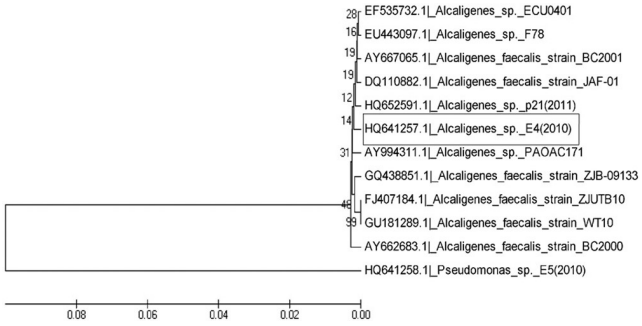

Fig. 1 — Phylogenetic tree expressing the relationships of identified endophytic antagonistic bacterial strains based on the 16S rDNA sequences. [The scale bar is in fixed nucleotide substations per sequence position. *Alcaligenes* sp. EIL-2 was used in this study; the remaining are database reference strains]

***In silico* studies of peroxidase protein and its possible interaction with substrates (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) in lignin biosynthesis pathway**

The amino acid sequence obtained by translation of the nucleotide sequence of *H. brasiliensis* peroxidase gene has 346 amino acids. The physicochemical properties showed that the peroxidase had a molecular weight of 37.07 kD and the theoretical pI value of 4.67. Total number of negatively charged and positively charged residues in peroxidase was 31 and 19, respectively. According to the model, it was predicted that the secondary structure of peroxidase is inclusive of 36.42% α-helix, 4.91% β-turns and 58.67% random coil. The SignalP-4.0 prediction showed that the peroxidase protein was secretory in nature and a cleavage site existed between amino acid position 27 and 28. Phylogenetic tree constructed on the basis of

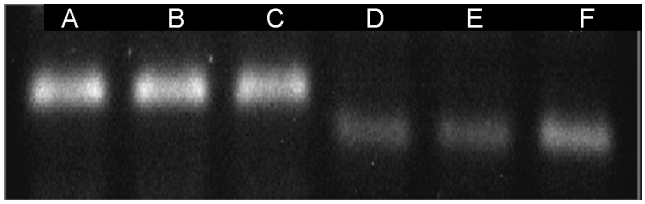

Fig. 2 — Gel photograph showing the amplification of endogenous control β-actin and peroxidase gene in antagonist inoculated and control plants of *H. brasiliensis.* (A) β-actin in un treated control; (B) β-actin in media inoculated plants; (C) β-actin in antagonist EIL-2 broth inoculated plants; (D) peroxidase in untreated control; (E) peroxidase in media inoculated plants; and (F) peroxidase in antagonist EIL-2 broth inoculated plants

Table 1—Biochemical estimation of β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase activity in antagonist treated and control plants of *H. brasiliensis*

Treatments	β-1,3-glucanase activity (μg glucose released min^{-1} g^{-1} tissue)				Peroxidase activity (U/L)				Phenylalanine ammonia lyase activity (nmol trans-cinnamic acid min^{-1} g^{-1} tissue)			
	0h	48h	96h	144h	0h	48h	96h	144h	0h	48h	96h	144h
Untreated control	30.56 ±1.94	30.68 ±2.35	35.25 ±3.35	33.94 ±2.70	73.54 ±6.48	69.25 ±8.24	63.18 ±5.49[b]	69.59 ±6.10[b]	40.80 ±2.24	38.89 ±2.05	40.98 ±2.27	40.27 ±1.98
Application of media alone	31.64 ±2.21	33.00 ±2.65	35.56 ±3.99	35.12 ±3.40	76.40 ±4.95	67.22 ±7.32	63.67 ±5.83[b]	70.81 ±6.51[b]	40.86 ±1.78	39.11 ±2.15	38.89 ±2.40	41.48 ±2.22
Application of antagonist EIL-2 broth	31.74 ±1.37	33.24 ±2.73	35.77 ±2.17	37.31 ±2.98	74.36 ±6.81	130.85 ±5.52	134.36 ±8.58[a]	100.44 ±8.98[a]	41.27 ±1.94	40.36 ±2.51	41.52 ±2.70	40.94 ±2.59

[Values are means ±SE. In peroxidase activity means ±SE followed by the same letter are not significantly different at P <0.05 according to Duncan's multiple range test]

amino acid sequence alignment revealed the similarity of sequence with other plant peroxidases (Fig. 3).

The three dimensional structure of class III peroxidase, from *H. brasiliensis* and its active site residues is given in Fig. 4. The peroxidase protein (PDBID: 1pa2A) from *Arabidopsis thaliana* with 66% sequence identity was used as template for modelling. A Q MEAN Z- score value of –1.706 showed the reliability of the model. The stereochemistry of the modelled structure was checked by the Ramachandran plot in which 89.3% of the total amino acids were present in most favoured regions and 10.3% in allowed regions (Fig. 5).

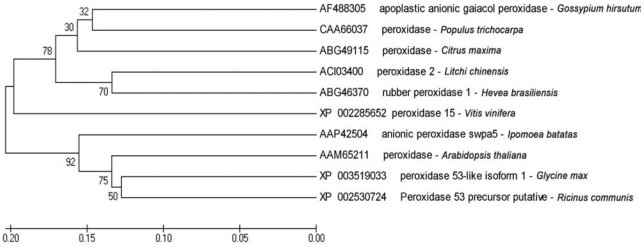

Fig. 3 — Dendrogram representing the amino acid sequence homology of peroxidase from *H. brasiliensis* with other plant species

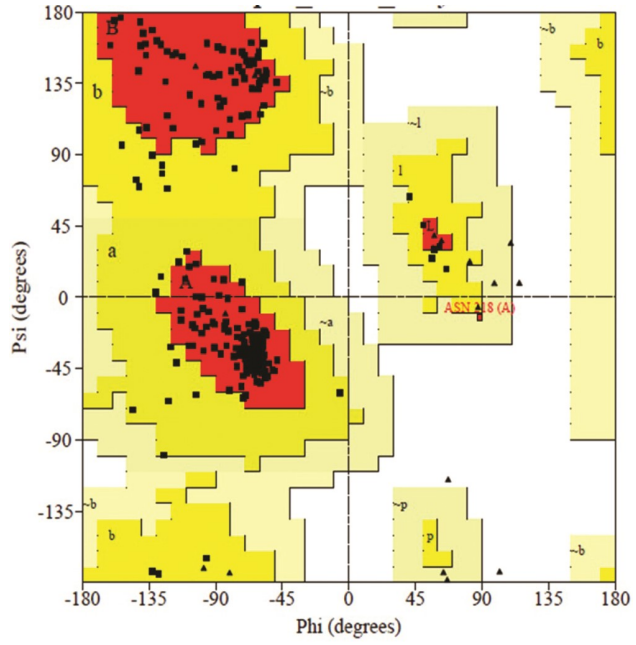

Fig. 4 — Homology modeled structure of the *H. brasiliensis* class III peroxidase protein. Active site residues are labelled

Docking studies showed that the monolignols were perfectly bound with active site of modelled *H. brasiliensis* peroxidise. Monolignols [coniferyl (CA), sinapyl (SA) and *p*-coumaryl alcohol (*p*-CA)] have almost similar binding energies with the modelled enzyme. The list of hydrogen bonding and Van der Waals (heavy atoms only) interactions between the monolignols and the peroxidase protein are given in Table 2. The modelled peroxidase *p*-coumaryl alcohol complex was stabilized by 53 Van der Waals contacts and one hydrogen bond formed by the amino acid residue Phe 199. The peroxidise coniferyl alcohol complex was stabilised by 80 Van der Waals contacts and 2 hydrogen bonds formed by the amino acid residues Ala 196 and Phe 199. The hydrogen bonding interactions were not observed in peroxidise sinapyl alcohol complex but Van der Waals contacts were 79. The hydrogen bonding interactions between the substrates and the modeled peroxidase are shown in Fig. 6.

Discussion

Abnormal leaf fall (ALF) caused by *Phytophthora meadii* is the most destructive disease of rubber in India. The disease occurs annually during southwest monsoon months, June to August. It infects pods, leaves and tender shoots causing heavy defoliation and dieback of tender twigs. Biological control of

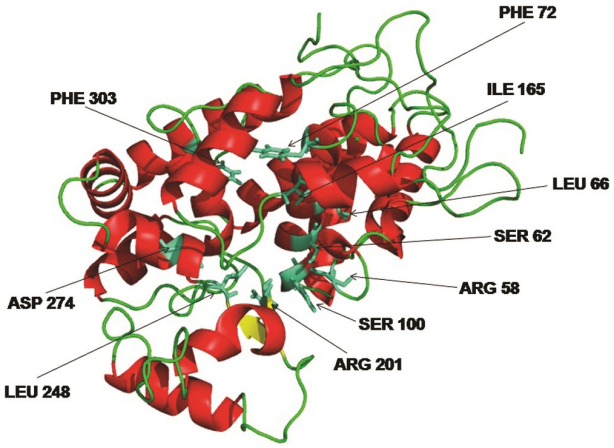

Fig. 5 — Ramachandran plot showing the phi/psi distribution of modeled structure of the peroxidase protein. [Red coloured regions represent the most favoured regions and the yellow regions represent the allowed regions]

Table 2—Docking features of modelled peroxidase with substrates of lignin biosynthesis pathway

Properties	Modeled peroxidase with p-coumaryl alcohol	Modeled peroxidase with coniferyl alcohol	Modeled peroxidase with sinapyl alcohol
Binding energy (kcal/mol)	-26.79	-26.29	-26.74
No. of hydrogen bonds	One	Two	Nil
Residues forming hydrogen bonds	Phe199	Phe199,Ala196	Nil
No. of Van der Waals interactions	53	80	79

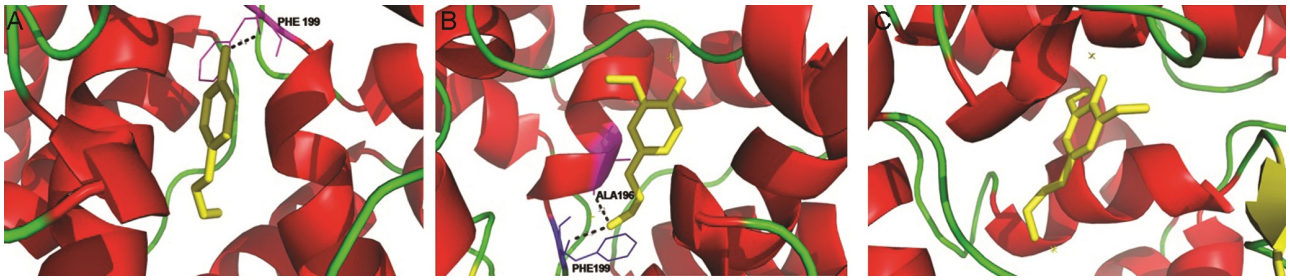

Fig. 6 — Hydrogen bond interactions of substrates in the lignin biosynthesis with catalytic residues in the active site of modeled peroxidase. Orientations of (A) p-Coumaryl alcohol; (B) Coniferyl alcohol; and (C) Sinapyl alcohol molecule in the active sites of peroxidise are different

diseases in plants is ecofriendly and is a potential component of integrative pest management (IPM). Unfortunately, several uncontrollable environmental factors, such as soil composition, pH, water availability, mineral and carbon source availability and potential competitive interactions with native microflora affect biological control agent (BCA) efficacy[30]. Therefore, if biological control is to become a viable alternative for crop protection, BCAs with multiple modes of action is ideal.

Microbial endophytes, which live in the intercellular spaces of living plant tissues, are relatively untapped source of microbial diversity for use in agriculture[31]. Endophytic bacteria live in plant tissues without causing substantive harm to plant. The internal tissue of plants provides a uniform and safe environment when compared to rhizosphere and phylloplane where the introduced bacterial population must compete for nutrients and also tolerate temperature changes and exposure to ultra violet (UV) rays. In this study, bacterial endophytes were isolated from *Hevea brasiliensis* and the antagonistic potential of these endophytes against *P. meadii* was evaluated. Out of 252, 42 isolates inhibited the growth of *P. meadii* under *in vitro* conditions. Six isolates were selected based on the higher percentage of inhibition on *P. meadii* and morphological differences. Among the six endophytes, A2L-4, A1P-1, A2R-1 and A1L-4 were from highly disease prone area, B2L-10 from moderately disease prone area, RRII farm, Kottayam and E1L-2 was from disease free area. The EIL-2 isolate was used for further studies and identified as *Alcaligenes* sp. (Fig. 1). *Alcaligenes* sp. was reported as endophytes from various plant species. You & Zhou[32] reported the intracellular colonization of *A. faecalis* in rice seedlings.

Guetsky *et al*.,[33] reported deployments of several BCAs with multiple modes of action, including production of antibiotics and induction of resistance, increases BCA efficacy by excluding the variability. Induced resistance has been studied for decades and defence-activating compounds are known as plant defence activators or systemic acquired resistance (SAR) inducers. Interestingly, induced systemic responses are not just initiated by pathogens and chemicals but also may result from interactions with avirulent microbes. The PR proteins are thought to play an important role in resistance and have been taken as a marker of the induced state in plants[34,35]. The potential of antagonistic bacteria has been correlated with alterations in the expression pattern of the PR genes[12,36]. Pre-treatment of susceptible apple cultivars with bacterium *P. fluorescens* Bk3 makes the plants more resistant to infection by the fungus *V. inaequalis*[37]. Transient increases in chitinase and peroxidase activities have been detected in leek roots during early stages of colonization by vesicular arbuscular fungi. The present study examined the activities of β-1,3 glucanase, peroxidase and PAL in popular clone (RRII 105) of *H. brasiliensis* after the application of an antagonistic bacterium. The variations of β-1,3-glucanase and PAL activity was not observed in leaf tissues after the application of antagonistic bacterium. The increased activity of peroxidase was estimated in the leaves of antagonist treated plants relative to non-treated ones as early as 48 h of post inoculation (Table 1).

Liu *et al*.,[38] demonstrated that leaf application of plant growth promoting rhizobacteria strain effectively induced resistance against leaf pathogens. Plants can detect the presence of molecules from bacteria through chemo-perception systems. Flagellin acts as an elicitor in whole *Arabidopsis thaliana* plants, inducing defence signalling and leading to the induction of defence related proteins[39]. The set of PR proteins induced in plants vary depends on the external stimuli. The application of antagonistic *Alcaligenes* sp. EIL-2 could not stimulate the

induction of PR proteins such as β-1,3-glucanase and PAL in *H. brasiliensis*. Production of PR proteins was compared in different clones of *H. brasiliensis* and variability in PR protein activity was observed among different clones during pathogenesis[40]. The observations clearly pointed out that each PR-protein in *H. brasiliensis* responds differentially to the application of the antagonistic bacterium EIL-2. The increase in peroxidase enzyme activities in the leaves suggests a defence response to the application of antagonistic bacterium in *H. brasiliensis*.

Recognition of the presence of microorganisms is the first step for activation of defence responses. Upon contact with pathogens or with non-pathogenic microorganisms or elicitors, the processes like ion fluxes, phosphorylation/dephosphorylation of proteins and production of signalling molecules, such as salicylic acid, jasmonic acid, ethylene and reactive oxygen species are activated. This leads to regulation of gene expression and induction of defence responses[41]. In sugarcane, analysis of genes expressed in response to the endophytic colonization revealed that inoculation with *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans* induced the genes for nitrogen assimilation and for carbon metabolism, favouring plant growth, as well as genes for limited plant defence mechanisms[42]. The present study revealed the induction of peroxidase level in antagonist treated *H. brasiliensis* clone RRII 105. The molecular studies evaluated the induction of class III peroxidase in *H. brasiliensis* which is specifically involved in lignifications process in plants. The study revealed over 2-fold increase in the peroxidase transcripts level in clone RRII 105 within two days of antagonist treatment (Fig. 2). It is assumed that the induced peroxidase could effectively induce the defence pathways in host plant and that may protect the plant from *P. meadii* invasion.

Peroxidase is involved in lignifications and leading to disease resistance[43]. It is considered as first defence barrier against successful penetration of invasive pathogens. Anatomical and cytological changes in tissues of bean seedlings have been reported in response to treatment of a nonpathogenic bacterial isolate prior to their inoculation with a virulent isolate of *R. solani*. Colonization of epidermal cells by the isolate promoted the deposition of an electron-dense cell wall material rich in lignin, suberin and phenolic compounds. The study also investigated the role of bacterial isolate as an inducing agent of systemic resistance by analyzing the changes in peroxidases, which catalyze the final polymerization step of lignin synthesis[44]. In the present *in silico* study, various bioinformatic tools were used to determine whether the observed biochemical changes of *Hevea* peroxides enzyme leading to structural modifications like lignifications. Typically, class III peroxidases are able to oxidize a wide variety of small phenolic compounds, including the lignin monomers, monolignols. The sequence for the homology modeling of *H. brasiliensis* class III peroxidase gene obtained from NCBI GenBank (DQ650301) and the same sequence was used for primer synthesis in Real-Time PCR studies. The Open Reading Frame (ORF) of the peroxidase gene was found to be 1041 bp and produce a protein with 346 amino acids. The signal peptide in the amino acid sequence of *Hevea* peroxidase was evaluated and a 27 amino acid peptide was observed in the N-terminus and confirmed its secretory nature. Phylogenetic tree showed the distinct difference in the amino acid sequence of *Hevea* peroxidase from other plant peroxidases and the alignment revealed the high similarity of this sequence with *Litchi chinensis* peroxidase. The secondary structure prediction showed higher α-helix, 36.42%, over other regular secondary structures, which is a common feature of peroxidase.

Lignin polymerization occurs *via* oxidative radicalization of monolignol phenols (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol), followed by combinatorial radical coupling. The construction of a three-dimensional model of a protein structure provides crucial insights to its structure and protein-ligand interactions involved in ligand binding, ligand stabilisation and substrate conversion. An atomic resolution model of a protein was constructed by homology modeling from amino acids of query sequence and the active site region was also predicted (Fig. 4). The stochiometric and energetic properties of the model was estimated and the results indicated that the model was geometrically reliable. In order to understand the possibility and nature of interactions of the substrates of lignin biosynthesis pathway with the target peroxidase computer aided docking studies were carried out (Fig. 6). The substrate-protein complex is stabilized by hydrogen bonds and many van der Waals interactions in the proteins (Table 2). The *in silico* study revealed that the modelled peroxidase can effectively interact with the substrates of lignin biosynthesis pathway for catalysis. The

interaction between the peroxidase enzyme and monolignols (substrates) proposed in this study are useful for understanding the potential mechanism of enzyme and substrate binding in lignifications process. The study concluded that lignifications through antagonistic endophyte induced peroxidase could be one of the pathways to induce resistance in *H. brasiliensis*.

In recent years, interest in the ability of beneficial microorganisms to induce resistance in plants has grown, particularly with respect to their use as environmentally safe controllers of plant diseases. Among these microorganisms, antagonistic endophytes have received only little attention as potential inducers of resistance. This study stated that the induction of pathogenesis-related proteins caused by the application of nonpathogenic antagonists may become an alternative method to the chemical control of plant diseases.

Conclusion

Inducing a plant's defence mechanism by prior application of a biological agent is an eco-friendly strategy in plant disease management. The present study evaluated the plant defence activation potential of an antagonistic endophyte from *H. brasiliensis*. The increased activity of pathogenesis related peroxidise protein was estimated in the leaves of antagonist-treated plants of *H. brasiliensis*. Lignification is a disease resistance mechanism in plants through class III peroxidases and it is considered as first defence barrier against successful penetration of invasive pathogens. Quantitative PCR estimation also showed 2.5-fold increase of class III peroxidase transcripts in antagonist treated plants. The *in silico* studies of induced class III peroxidase confirmed its efficiency in interaction with the substrates of lignin biosynthetic pathway for catalysis. The present study highlighted the potential of antagonistic endophyte as a bio-agent for inducing plants defence mechanism and can play a significant role in sustainable disease management.

Acknowledgement

The first author Amith Abraham acknowledges Kerala State Council for Science, Technology and Environment (KSCSTE), India for providing Post Doctoral Fellowship; and the 5th author Raveendran Sindhu acknowledges Department of Science and Technology, Ministry of Science & Technology, Govt of India for financial support under DST WOS-B Scheme.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1 Anderson AJ, Blee KA & Yang KY, Commercialisation of plant systemic defence activation: theory, problems and successes. In: *Multigenic and Induced Systemic Resistance in Plants*. (Ed. S Tuzun & E Bent, Springer, Berlin), 2006, 386.

2 Van Loon LC, Bakker PAHM & Pieterse CMJ, Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol*, 36 (1998) 453.

3 Flor H, Current status of the gene-for-gene concept. *Annu Rev Phytopathol*, 9 (1971) 275.

4 Sticher L, Mauch-Mani B & Me´ traux JP, Systemic acquired resistance. *Annu Rev Phytopathol*, 35 (1997) 235.

5 Lawton MA & Lamb C, Transcriptional activation of plant defence genes by fungal elicitors, wounding and infection. *Mol Cell Biol*, 7 (1987) 335.

6 Kuc J & Rush JS, Phytoalexins. *Arch Biochem Biophys*, 236 (1985) 455.

7 Hammerschmidt R, Nuckles EM & Kuc J, Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol Plant Pathol*, 20 (1982) 73.

8 De Meyer G. & Hofte M, Salicylic acid produced by rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induced resistance to leaf infection by *Botrytis cinerea* on bean. *Phytopathology*, 87 (1997) 588.

9 Leeman M, den Ouden FM, van Pelt JA, Dirkx FPM, Steijl H, Bakker PAHM & Schippers B, Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. *Phytopathology*, 86 (1996) 149.

10 Bargabus RL, Zidack NK, Sherwood JE & Jacobsen BJ, Characterization of systemic resistance in sugar beet elicited by a non pathogenic, phyllosphere colonizing *Bacillus mycoides*, biological control agent. *Physiol Mol Plant P*, 61 (2002) 289.

11 Saravanakumara D, Charles V, Kumarb N & Samiyappan R, PGPR-induced defence responses in the tea plant against blister blight disease. *Crop Prot*, 26 (2007) 556.

12 Park K & Kloepper JW, Activation of PR-1a promoter by rhizobacteria that induce systemic resistance in tobacco against *Pseudomonas syringae* pv. tabaci. *Biol Control*, 18 (2000) 2.

13 Compant S, Reiter B, Sessitsch A, Nowak JC & Barka EA, Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Appl Environ Microbiol*, 71 (2005) 1685.

14 Métraux JP & Boller T, Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial and fungal infections. *Physiol Mol Plant Pathol*, 28 (1986) 161.

15 Broglie K, Chet I, Holliday M, Cressman, R, Biddle P, Kowlton S, Mauvais C & Broglie R, Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science*, 254 (1991) 1194.

16 Van Loon LC & Van Strien EA, The families of pathogenesis-related proteins, their activities, and

comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol*, 55 (1999) 85.

17 Tamura K, Dudley J, Nei M & Kumar S, MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol*, 24 (2007) 1596.

18 Sneath PHA & Sokal RR, Numerical Taxonomy, Freeman, San Francisco, 1973.

19 Felsenstein J, Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39 (1985) 783.

20 Pan SQ, Ye X.S & Kuc J, Association of β-1, 3- glucanase activity and isoform pattern with systemic resistance to blue mould in tobacco induced by stem injection with *Perenospora tubacina* or leaf inoculation with tobacco mosaic virus. *Physiol Mol Plant Pathol*, 39 (1991) 25.

21 Putter J, Peroxidase, In: *Methods of Enzymatic Analysis*, Vol. II. (Ed. HU Bergemeyer, Academic Press, London), 1974,685.

22 Bruske CH, Phenylalanine ammonia lyase activity in tomato roots infected and resistant to the root-knot nematode (*Meloidogyne incognita*). *Physiol Plant Pathol*, 16 (1980) 409.

23 Petersen TN, Brunak S, von Heijne G & Nielsen H, SignalP 4.0: discriminating signal peptides from trans-membrane regions. *Nat Methods*, 8 (2011) 785.

24 Arnold K, Bordoli L, Kopp J & Schwede T, The SWISS-MODEL workspace: a web-based environment for protein structure homology modeling. *Bioinformatics*, 22 (2006)195.

25 Guex N & Peitsch MC, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*, 18 (1997) 2714.

26 Laskowski RA, MacArthur MW, Moss DS & Thornton JM, PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Cryst*, 26 (1993) 283.

27 DeLano WL, *The PyMOL Molecular Graphics System.* DeLano Scientific, San Carlos, CA, USA, 2002.

28 Laurie AT & Jackson RM, Q-Site Finder: an energy based method for the prediction of protein-ligand binding sites. *Bioinformatics*, 21 (2005) 1908.

29 Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS & Olson AJ, AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *J Comput Chem*, 30 (2009) 2785.

30 Paulitz TC & Baker R, Biological control of *Pythium* damping-off of cucumbers of *Pythium nunn*: Influence of soil environment and organic amendments. *Phytopathology*, 77 (1987) 341.

31 Bacon CW & White JF, Physiological adaptations in the evolution of endophytism in the Clavicipitaceae, In: *Microbial Endophytes* (Ed. CW Bacon & JF White, Marcel Deker, NY), 2000, 237.

32 You CB & Zhou FY, Non-nodular endorhizospheric nitrogen fixation in wetland rice. *Can J Microbiol*, 35 (1989) 403.

33 Guetsky R, Shtienberg D, Elad Y, Fischer E & Dinoor A, Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology*, 92 (2002) 976.

34 Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Metraux JP & Ryals JA, Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell*, 3 (1991) 1085.

35 Kessmann H, Staub T, Ligon J, Oostendorp M & Ryals J, Activation of systemic acquired disease resistance in plants. *Eur J Plant Pathol*, 100 (1994) 359.

36 Ramamoorthy V, Raguchander T & Samiyappan R, Induction of defence-related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. *Lycopersici*. *Plant Soil*, 239 (2002) 55.

37 Kucheryava N, Fiss M, Auling G & Kroppenstedt RM, Isolation and characterization of epiphytic bacteria from the phyllosphere of apple, antagonistic *in vitro* to *Venturia inaequalis*, the causal agent of apple scab. *Syst Appl Microbiol*, 22 (1999) 472.

38 Liu L, Kloepper JW & Tuzun S, Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth promoting bacteria. *Phytopathology*, 85 (1995) 843.

39 Boller T, Chemoperception of microbial signals in plant cells. *Annu Rev Plant Physiol Plant Mol Biol*, 46 (1995) 189.

40 Thanseem I, Joseph A & Thulaseedharan A, Induction and differential expression of β-1,3-glucanase mRNAs in tolerant and susceptible *Hevea* clones in response to infection by *Phytophthora meadii. Tree Physiol*, 25 (2005) 1361.

41 Garcia-Brugger A, Lamotte O, Vandelle E, Bourque S, Lecourieux D, Poinssot B, Wendehenne D & Pugin A, Early signalling events induced by elicitors of plant defences. *Mol Plant Microbe In*, 19 (2006) 711.

42 De Matos Nogueira E, Vinagre F, Masuda HP. Vargas C, de Padua VLM, da Silva F. R, dos Santos RV, Baldani JI, Ferreira PC & Hemerly AS, Expression of sugarcane genes induced by inoculation with *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans*. *Genet Mol Biol*, 24 (2001) 199.

43 Lagrimini LM, Burkhart W, Moyer M & Rothstein S, Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue specific expression. *Proc Natl Acad Sci USA*, 84 (1987), 7542.

44 Jabaji-Hare SH, Chamberland H, Charest PM & Echandi E, *Cell wall alterations in bean seedlings protected from Rhizoctonia root rot by binucleate Rhizoctonia species*. (Abstract Book of the Proceedings for the 6th International Congress of Plant Pathology), 1994, 288.